The Phenotypic Response of Dendritic Cells to Gold Nanoparticles Treatments

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By

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The Phenotypic Response of Dendritic Cells to Gold Nanoparticles Treatments

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Introduction

Immunotherapy research has been increasingly investigating the potential of gold nanoparticles (AuNPs). AuNPs pose new benefits in the medical field ranging from diagnostics to diseases treatment. AuNPs' ability to infiltrate tissue and target immune cells makes their potential highly useful for new proposed personalized immunotherapies[1] regarding antigen specific targeting delivery, tracking capabilities *in vivo* [2][3], and more effective and direct vaccines [8]. AuNPs act as an adjuvant with the ability to elicit immuno-suppressive or immuno-activated responses depending on the treatment and characterization of the AuNPs. A promising application of AuNPs is their ability to interact with dendritic cells (DCs). DCs are antigen presenting cells (APCs) and play an integral part in both innate and adaptive immune responses. They work by internalizing and presenting antigens on their surface to other immune cells initiating an immunomodulatory response. In previous research, it has been shown that AuNPs engineered with surface molecules can the initiate maturation of immature DCs (iDCs). Depending on the surface molecules, AuNPs can mature iDCs to become either activated or tolerogenic DC phenotypes[1]. These matured DC phenotypes use the AuNP's surface molecules to then elicit an immune response by presenting the surface molecules to other immune cells in addition to secreting chemokines and cytokines to enhance the immune response. Though AuNPs' influence on the maturation of iDCs has been increasingly studied, it is still not well understood which is critical in order to develop effective personalized immunotherapies.

In this study, the relationship between DC phenotypes and AuNP properties is analyzed in order to optimize the methods used to elicit specific immune responses. iDCs will be cultured and treated with AuNPs with various surface modifications which will then be analyzed to determine the phenotypic character of the cultured DCs. The cultured DCs are analyzed using

high-throughput screening and flow cytometry to determine the surface molecules that have developed from the AuNP treatment which will determine which phenotype of the matured iDCs. This analysis will establish a relationship between various AuNP treatments and the resulting phenotypic development of DCs. This research will work towards standardizing maturation methods of DCs *in vivo* in order to control a patient's immune system and its responses to fight off diseases and arm immune cells.

Literature Review

1. Innate and Adaptive Immunity

The immune system is categorized into two distinct parts, the innate and adaptive immune responses. The innate immune response provides an immediate but non-specific defense. Adaptive immunity is a slower defense system, however, is it more specific and precise in its mechanism. This immunity undergoes antigen specific responses through the use of T and B lymphocytes while also creating a memory of the response in order to have a rapid and powerful response in subsequent exposures[4]. The bridge between innate and adaptive immune responses is dendritic cells (DCs). In order to understand the initiation and propagation of these responses, it is important to understand the mechanism behind DC function.

2. Dendritic Cells

Dendritic cells are powerful antigen presenting cells (APCs) that activate and mediate a host immune responses. A single DC is capable of activating hundreds of T cells making them an integral part in both adaptive and innate immune responses. DCs have two primary functions: to capture and present antigens to other immune cells in order to elicit a response. DCs accomplish

this goal by phagocytizing the foreign body, processing them within the cell, and presenting the corresponding antigen peptide on the surface of the cell. The ability of DCs to present specific antigens to activate targeted T cell responses has shown to have potential in new vaccine strategies for various infections, allergies, autoimmune diseases, cancer, and a variety of illnesses[5].

DCs develop originally from myeloid progenitor cells within the bone marrow which have the ability to differentiate into monocytes. Monocytes are produced naturally at a cost rate and circulate throughout the bloodstream until undergoing spontaneous apoptosis[6]. When the innate immune response is triggered, the circulating monocytes are recruited by cytokine proteins that are secreted by leukocytes at the site of trauma/inflammation[7]. Once the monocytes are successfully recruited to the site, they differentiate into immature DCs (iDCs) when in the presence of interleukin-4 (IL-4) and granulocyte-macrophage colony-stimulating factor (GM-CSF)[8]. These iDCs phagocytize antigens at the inflammation site and produce the corresponding peptides that are capable of binding to major histocompatibility complex class I and II (MHC I and MHC II) which correspond to cytotoxic T cells and helper T cells activation, respectively. During this process, the DCs' phenotype changes from immature to mature DCs (mDCs). Once this phenotypic change has occurred, the mDCs lose their affinity towards the inflammatory cytokines and instead increase their affinity for lymphoid cytokines[9]. This causes them to migrate to the draining lymph nodes where T and B lymphocytes reside waiting to be prompted by APCs to elicit immune-activating or immune-suppressive response.

3. Gold Nanoparticles

Nanoparticles (NPs) are defined as an object within the range of 1-100nn in size. The function and use of nanoparticles vary with material, size, shape, surface additions, surface charges, and concentration. In regards to immunotherapy, gold nanoparticles (AuNPs) in particular have been found to be effective due to their stability, solubility, control over size of particles, and ability to attach surface molecules, the latter of which is particularly important[1]. This ability can be used to make AuNPs function as an antigen carrier which can stimulate antigen presentation when phagocytized by iDCs. Using different surface coatings, the maturation of the iDCs can by controlled using certain antigens which can initiate iDCs to mature into tolerogenic DCs (tDCs) and have an immune-regulatory or suppressive response rather than immune-activating response. However, this process is not well understood and requires more optimization with AuNP concentration and surface coating composition.

AuNPs have been shown to migrate to the lymph nodes when between the range of 20-200nm[10]. Within this range, larger sizes showed decreased amount of captured AuNPS. However, lower sizes were shown to be effective too increased expression of proinflammatory genes as well cytotoxicity in comparison to silver NPs. Through optimization, 60nm AuNPs were determined to be the most effective comprimise[11,12,13].

Though AuNP size has been optimized, concentration and surface coating have not been. Increasing concentration of AuNPs have been shown to correlate with increased cytotoxicity, where low concentrations have shown lower concentration of captured particles[14,15]. In addition, surface chemistry has been studied in regard to uptake, however, strong correlations between uptake and maturation and surface chemistry has not been established[16]. In order to develop an optimized process by which iDCs can be controlled to mature into either mDCs or tDCs, more

research must be done in optimizing the concentration and surface chemistry of the AuNPs used to influence iDCs.

Materials and Methods

These methods are adapted and modified from Kou PM and Babensee JE.[17]

1. Human Serum Extraction

On day 0 of DC, human peripheral blood was collected from consenting volunteers at the Student Health Phlebotomy Laboratory in accordance with protocol H10011 of H15072 approved by the Institutional Review Board of the Georgia Institute of Technology. Once collected, the blood clotted the supernatant was extracted and for future use on day 5 of DC culture. This supernatant contains the donors serum.

2. Derivation of immature DCs (iDCs)

A second sample of human peripheral blood was collected from the same consenting volunteers at the Student Health Phlebotomy Laboratory in accordance with protocol H10011 or H15072 approved by the Institutional Review Board of the Georgia Institute of Technology. The whole blood was heparinized and diluted with phosphate buffered saline (PBS). Peripheral mononuclear cells (PBMCs) were then isolated using centrifugation with lymphocyte separation medium (LSM). Following centrifugation, yellow supernatant was aspirated. The buffy coat and remaining clear liquid was extracted and added to a PBS tube then centrifuged and the supernatant aspirated. The cell were resuspended in lysis buffer and combined into one tube. After several washes using PBS, the PBMCs were resuspended at a concentration of $5x10^6$ cells/ml in DC media, comprised of filter-sterilized RMPI-1640, heat inactivated fetal bovine

serum, and Penicillin/Streptomycin. The cells were then plated in a volume of 10 ml/plate in a tissue-culture dish. They were then incubated for 2 hours at 95% relative humidity and 5% CO_2 at 37◦C. Following this period, the culture dishes were washed three times with warm DC media to remove non-adherent cells from culture. New warmed media was added to the cell culture plates with the remaining adherent cells at a volume of 10ml/plate, and supplemented with the growth hormones GM-CSF and IL-4. The cells were then finally incubated for a period of 5 days at 95% relative humidity and 5% CO_2 at 37◦C. During this culture time, this will induce the differentiation of monocytes into iDCs.

3. AuNP treatments preparation

Gold nanoparticles (60 nm) were kindly supplied by the University of Toronto as stock solutions containing uncoated (bare), PEG-2K coated and PEG-5K coated AuNPs. All stock solutions had an AuNP concentration of 5 nM. The bare AuNPs were suspended in ultrapure water and the PEGylated AuNPs were suspended in PBS in their respective stock solutions.

A. Bare, PEG-2K and PEG-5K coated AuNP treatment preparation

32 μL of each stock solution were aliquoted; two aliquots of the bare AuNP stock were prepared, one for the final bare AuNP treatment and one for the final serum-coated AuNP treatment. The four aliquots were then centrifuged in order to form a pellet of the AuNPs. Each aliquot was then washed with sodium citrate tribasic dehydrate solution twice followed by centrifuging the samples and another aspiration of the supernatant. The AuNP pellets of a bare AuNP aliquots, the PEG-2K AuNP aliquot, and the PEG-5K AuNP aliquot were resuspended in RPMI media. This preparation was for an AuNP treatment sample concentration of 160 pM. In

order to create the solutions for the other treatments, serial dilutions were performed on the three aliquots to obtain three concentrations (160 pM, 16 pM, and 1.6 pM) for each AuNP coating type.

B. Serum-coated AuNP treatment preparation

The pellet in the second bare aliquot was resuspended within chilled PBS and added to human serum in an Eppendorf tube (filtered via 0.22 μm PES filter). The solution was incubated in human serum for 1 hour with 95% relative humidity and 5% CO_2 at 37◦C. After the incubation period, the serum-coated AuNPs solution was centrifuged followed by the supernatant being aspirated. The serum-coated AuNPs were then washed with chilled PBST twice by adding chilled PBST to the AuNPs pellet . This solution was then centrifuged with another aspiration of the supernatant. After the second PBST wash, the serum-coated AuNPs were washed with chilled PBS by the same process. The final serum-coated AuNP pellet was resuspended DC media for an AuNP treatment sample concentration of 160 pM. A serial dilution was performed to obtain three concentrations (160 pM, 16 pM, and 1.6 pM) of serum-coated AuNP solutions.

4. Treatment of iDCs with AuNPs

On day 5, loosely adherent and non-adherent iDCs were harvested and resuspended in DC media with 1000 U/ml GM-CSF and 800 U/ml IL-4 at a cell concentration of $5x10^5$ DCs/ml. 100 μL of iDCs at a concentration of 5×10^5 DCs/ml were plated onto each well in the 96-well tissue culture plate. The wells for the negative control of iDCs remained untreated. The wells for the positive control of mDCs were treated with LPS. Lastly, the wells for the positive control of tDCs were treated with human IL-10 and human IFN-alpha. In accordance to Figure 1, 150 μl of

each concentration group for each AuNP coating treatment group were added to different wells of the 96-well plate containing iDCs, such that the final concentration of AuNPs in each well were 0.1 pM, 1.0 pM and 10 pM. The iDCs were then incubated with the control, bare AuNP, PEG-2K coated AuNP, PEG-5K coated AuNP, and serum coated AuNP treatments for 24 hours with 95% relative humidity and 5% CO_2 at 37◦C.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Antibody 150 ul/well iDC			Isotyp 150 ul/well iDC			Antibody 60nm - 150 ul 0.1pM Au(PEG5K)			Cells-Back-ground	Cells-Back-ground	Cells-Back-ground
B	Antibody 150 ul/well mDC			Isotyp 150 ul mDC			Isotyp 60nm - 150 ul 0.1pM Au(PEG5K)			Media	Media	Media
C	Antibody 150 ul/well tDC			Isotyp 150 ul tDC			Antibody 60nm - 150 ul 1pM Au(PEG5K)			Isotyp 60nm - 150 ul 1pM Au(PEG5K)		
D	Antibody 60nm - 150 ul 0.1pM Au			Isotyp 60nm - 150 ul 0.1pM Au			Antibody 60nm - 150 ul 10pM Au(PEG5K)			Isotyp 60nm - 150 ul 10pM Au(PEG5K)		
E	Antibody 60nm - 150 ul 1pM Au			Isotyp 60nm - 150 ul 1pM Au			Antibody 60nm - 150 ul 0.1pM Au(PEG2K)			Isotyp 60nm - 150 ul 0.1pM Au(PEG2K)		
F	Antibody 60nm - 150 ul 10pM Au			Isotyp 60nm - 150 ul 10pM Au			Antibody 60nm - 150 ul 1pM Au(PEG2K)			Isotyp 60nm - 150 ul 1pM Au(PEG2K)		
G	Antibody 60nm - 150 ul 0.1pM Au(Serum)			Isotyp 60nm - 150 ul 0.1pM Au(Serum)			Antibody 60nm - 150 ul 10pM Au(PEG2K)			Isotyp 60nm - 150 ul 10pM Au(PEG2K)		
H	Antibody 60nm - 150 ul 1pM Au(Serum)			Isotyp 60nm - 150 ul 1pM Au(Serum)			Antibody 60nm - 150 ul 10pM Au(Serum)			Isotyp 60nm - 150 ul 10pM Au(Serum)		

Figure 1. Layout of AuNP-DC treatments in 96-well plate. AuNP concentrations of 0.1 pM, 1.0 pM and 10 pM were added to DCs in individual wells for all AuNP treatment groups (bare, serum coated, PEG-2K coated, and PEG-5K coated AuNPs). Antibody staining was added to three of the six wells used for each treatment, and isotype staining was added to the other three wells.

On day 6, the DCs cultured in the 96-well plate were transferred directly to wells of a 96-well black filter plate that has been primed with PBS. The supernatants were then removed from the

individual wells by centrifuging the filter plate. After the supernatant is removed, 100 µl of cold working fixation solution (0.05 % paraformaldehyde) was added to each well. The plate was incubated for at least 30 min at room temperature on a microplate shaker at 600 rpm followed by the removal of the fixation solution through centrifugation. Following this, DCs were stained with antibodies for surface marker expression: anti-DC-SIGN-FITC, anti-CD86- PE, and anti-ILT3-AF647 (Figure 1). The following antibodies were used in isotype staining for background fluorescence elimination: IgG2B-FITC, IgG1-PE and IgG1κ (Figure 1).

The plate was then incubated in a plate shaker in the fridge covered in aluminum foil in order to be protected from light. After this staining protocol is completed, DCs were washed three times with FACS Buffer solution followed by centrifugation at 400 RCF for 4 min. Afterwards, the FACS Buffer solution was added to DCs. The 96 well plate was then read, and the fluorescence of each treatment group were measured. The geometric mean fluorescent intensities were measured with a Tecan Infinite F500 microplate reader using excitation filters of 535/25 and 485/20 and the emission filters of 590/20 and 535/25, for PE and FITC, respectively, and 650/668 for anti-ILT3 - AF647.

The surface marker, CD86, is a costimulatory molecule that doubles as a good indicator for the presence of mDCS due to upregulation once matured. Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin (DC-SIGN) is an endocytic receptor which expression is not significantly down-regulated upon DCs maturation. Immunoglobulin- like transcript 3 (ILT3) is an inhibitory receptors which is used as an indicator for the maturation of tDCs due to the expression of ILT3 being up-regulated upon anti-inflammatory DC response. The ratio of respective geometric mean fluorescent intensities for CD86 expression divided by DC-SIGN expression defines the metric of “inflammatory maturation factor” (IMF) as an

indicator of pro-inflammatory DC phenotype. The ratio of respective geometric mean fluorescent intensities for ILT3 expression divided by CD86 expression defines the metric of "tolerogenic maturation factor" (TMF) as an indicator of tDC phenotype.

Results

From the initial results (two donors), it was observed that the DCs treated with serum coated AuNPs would mature into tDCs. This was determined by comparing the control tDC wells to the serum treated wells. The DCs treated with the bare AuNPs also expressed characteristics of tDCs, however, only the serum treatment group had values higher than the control tDC as shown in Figure 2.

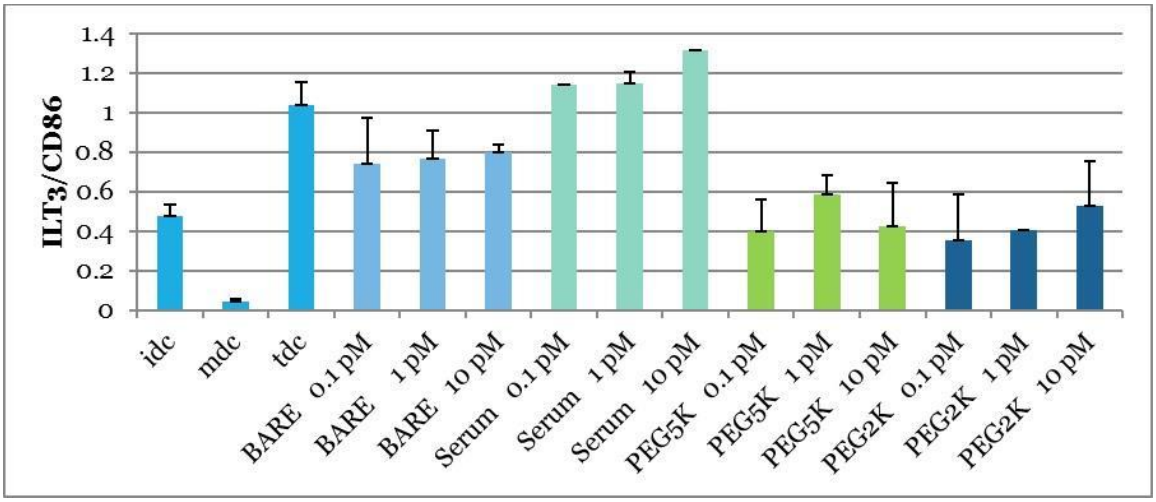

Figure 2. Tolerogenic maturation factor (ILT3/CD86) of dendritic cells in respect to treatment group and concentration. DCs were treated for 24 hours followed by antibody staining and isotype staining for ILT3 and CD86 to determine tolerogenic effects of each treatment group. The error bars represent the experimental range of the experiment.

The bare, PEG-2K, and PEG-5K coated AuNPs have shown to initiate a immuno-activated mDCs which, like the tDCs, was quantified using the relevant control well. An interesting result of these treatments was that none of the activated mDC levels were consistently higher than the respective control unlike with the tDC treatment (Figure 3). In addition to this,

the initial results showed that with some of the treatment groups that with increasing AuNP concentration, the respective differentiation will accordingly scale-up as seen in Figure 3. This was seen in the serum coated AuNPs in regard to tDC differentiation and seen in the bare and PEG-5K AuNPs in respect to mDC differentiation. As expected, the serum coated AuNPs had an inverse relationship between concentration and IMF as the DCs. As of now this is only speculation based off of initial testing, but following more trials this trend may become significant. Once all the results are acquired, trends between AuNP treatment and cell response through signaling and receptor development can be determined.

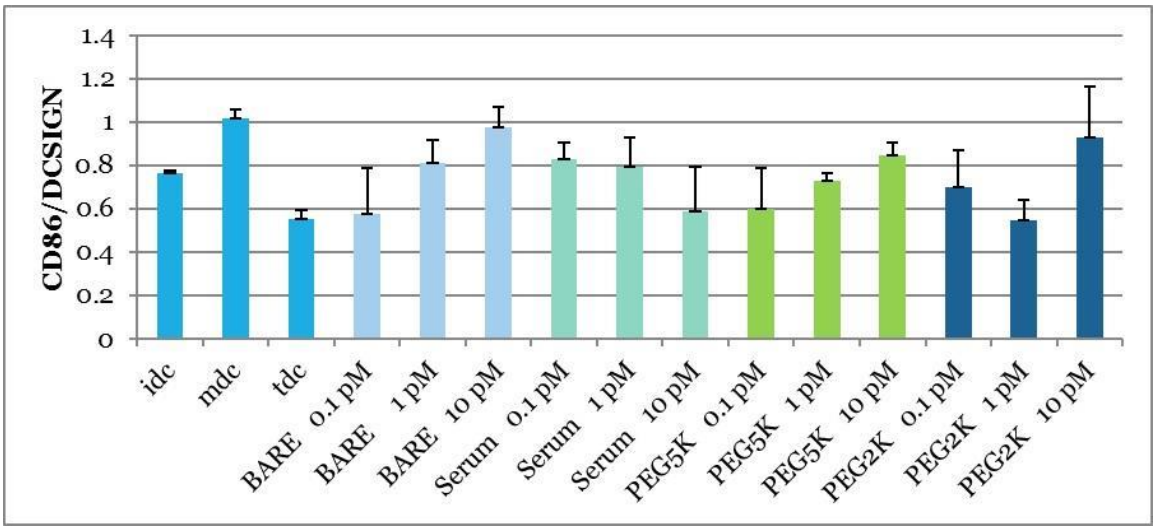

Figure 3. Inflammatory maturation factor (CD86/CDSIGN) of dendritic cells in respect to treatment group and concentration. DCs were treated for 24 hours followed by antibody staining and isotype staining for CD86 and DCSIGN to determine immune activating effects of each treatment group. The error bars represent the experimental range of the experiment.

Discussion

Each treatment group affected the phenotypic differentiation of DCs in a different way. Of all the treatment groups, the serum coated AuNPs had the highest levels of TMF with a slight positive trend between AuNP concentration and TMF. The data also suggests that the serum coated AuNPs are very effective at eliciting a tolerogenic response as the TMF levels were higher for the treatment groups than even the tDC control. However, the serum coated AuNPs

also tended to activate the DCs as seen by the levels of IMF. This effect may be minimized by using a higher concentration of AuNPs since the 10pM concentration had the lowest level of mDC activation while also having the highest level of tDC differentiation.

The PEG AuNPs had a very different response. Both the PEG-2K and PEG-5K had fairly low activation of tDCs and mDCs with the exception at high concentrations where they were seen to have higher mDC activation. The effects of the PEG-5K AuNPs towards mDC activation were seen to be concentration dependent, so there is a possibility that at higher concentrations the PEG-5K AuNPs would have a more significant mDC response. The same may be true with the PEG-2K AuNPs, however, the dependence on concentration is less apparent because the 1pM group had a lower activation of mDCs than the 0.1pM treatment group.

The bare AuNPs showed potential as a way to activate the immune system. It was observed that the bare AuNPs had the ability to make DCs change into mDCs at increasing concentrations with the 10pM having a IMF approximated equal to the mDC control. This means that AuNPs could potentially be used as a vehicle to activate DCs towards a certain antigen. Since the particle is enough to be recognized as a foreign body, any surface molecule or antigen on the AuNP's surface could also be associated with the activation effect of the AuNP. Through this mechanism, coated AuNPs could serve as a method to activate a native DCs *in vivo* towards a specific target.

Utilizing the same mechanism, AuNPs could potentially also act as a method to suppress the immune system towards certain targets. Instead of bare or PEG AuNPs, serum coated AuNPs could be used to elicit a tolerogenic response through the differentiation of iDCs into tDCs. When creating the serum coated AuNPs, other surface molecules could be added to the AuNPs

that may be associated with the tolerogenic response prompted by the serum coated. This could provide a novel strategy to combat autoimmune reactions.

The results of this study are fairly limited due to the low sample size (n=2). Further data acquisition is needed in order for these trends to be more defined and to demonstrate statistical significance. Another limitation of this study is that the mechanism of the action is assumed to be through DC phagocytosis of the AuNPs. Since this study utilizes *in vitro* cultures, these results could have been due to DC and AuNP interactions on the surface. In order to account for this, *in vivo* studies need to be conducted to show that AuNPs can be delivered and internalized by DCs. Additionally, the uptake of AuNPs by the DCs should be measured using an Inductively Coupled Plasma-Atomic Emissions Spectroscopy (ICP-AES) towards gold and magnesium content. This will allow for the mechanism for uptake to be characterized.

Conclusion

The initial results of this study demonstrate the potential for AuNPs to be used as a vehicle to activate or suppress the immune system through interaction with DCs. With the use of serum coated AuNPs, DCs can be manipulated into differentiating into tDCs where they can initiate an immune-suppressive response. This mechanism could lead to potential autoimmune therapies that teaches a patient's immune system to correct the incorrect response. AuNPs also indicated the ability to act as an immune-activating mechanism through the use of bare AuNPs. This could lead to methods of activating an *in vivo* immune response towards specific antigens that may otherwise go undetected like cancer. Though the initial findings show promise, further research needs to be done in order to prove the mechanism of AuNPs and their potential therapeutic effect.

Future Work

In the future, studies should be performed to further investigate the effect that AuNPs of various surface modifications have on DC behavior and mechanism by which different DC phenotypes are induced by AuNPs. Some immediate areas to research next include cytotoxicity analysis(CCK-8), cytokine profile analysis using Multiplex bead technology, and DC imaging to assess the extent of AuNP internalization using Inductively Coupled Plasma-Atomic Emissions Spectroscopy (ICP-AES) for gold and magnesium content. Additionally, the effects of AuNPs on other immune cells such as Tcells and macrophages should be investigated in hopes to more holistically understand the mechanism by which AuNPs influence an animals immune system. Lastly, *in vivo* studies need to be performed in order to prove the concept in a fully active system.

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